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Ecdysteroid receptors in *Drosophila melanogaster* adult females

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Summary

Ecdysteroid receptors were identified and partially characterized from total cell extracts of whole animals and dissected tissues from *Drosophila melanogaster* adult females. Binding studies indicated the presence of two ecdysteroid binding components having high affinity and specificity consistent with receptors previously identified in embryos and larvae. The highest affinity binding component in 3- to 4-day females had a dissociation constant of 9.2×10^{-10} M and a maximal binding concentration of approximately 90 pmol/g protein, with a lower affinity component having a dissociation constant of 2.94×10^{-9} M. Receptors at similar concentrations were also observed in abdominal walls containing adult fat body, with relatively lower receptor levels observed in ovaries. These results indicate that the observed ecdysteroid hormone concentrations in adult females can account for a physiological stimulatory effect on yolk protein synthesis in adult fat body.

Introduction

A major function of ecdysteroids in *Drosophila melanogaster* adults is the stimulation of yolk protein (YP) synthesis in the female adult fat body (Handler and Postlethwait, 1978; Jowett and Postlethwait, 1980). This effect can be experimentally stimulated by 10^{-8} M to 10^{-7} M 20-hydroxyecdysone (20-OH ecdysone) (Postlethwait and Handler, 1979), having a direct influence on yolk polypeptide transcript level (Shirk et al., 1983). Although it was discovered that circulating and whole animal ecdysteroid concentrations exist at these approximate levels in adult females (Handler, 1982; Bownes et al., 1984), these titers are nevertheless relatively low and close to basal concentrations observed earlier in development (Hodgetts et al., 1977; Kraminsky et al., 1980; Handler, 1982). The physiological significance of this hormone concentration on YP synthesis would be strengthened by determining the existence of ecdysteroid receptors in the fat body of adult females, having characteristics consistent with a saturation of binding sites at the physiological hormone concentration.

Ecdysteroid receptors have thus far not been identified in adults of *Drosophila* or other insect species, although they have been identified and characterized in several hormone-responsive tissues and cell types from embryos and larvae. These include *D. melanogaster* embryonic (Maroy et al., 1978; Sage et al., 1982) and tumorous

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(Dinan, 1985) cell lines, imaginal discs (Yund et al., 1978; Yund, 1979), larval fat body (Gronemeyer et al., 1983), whole embryos (Osterbur and Yund, 1983; Deak et al., 1988), and salivary glands (Schaltmann and Pongs, 1982), as well as in *Calliphora* whole larvae (Lehmann and Koolman, 1988). Recently, partial purification of ecdysteroid receptor from a *D. melanogaster* embryonic cell line has been reported (Landon et al., 1988).

In this report we identify and partially characterize for the first time ecdysteroid receptors in *D. melanogaster* adult females, localizing a large proportion of the total receptor to the adult fat body. These results support the physiological influence of previously observed 20-OH ecdysone titers in adult females on YP synthesis.

Materials and methods

Animal and tissue preparation

Drosophila melanogaster Oregon-R wild type or mutant strains were raised on standard cornmeal-agar-molasses media at 25 °C. Animals were collected within 12 h of emergence, sexed, and incubated for appropriate time periods. Tissue samples were dissected in ice-cold Drosophila ringers solution (Ephrussi and Beadle, 1936) and rinsed twice in homogenization buffer just previous to sample preparation, or stored in buffer with 10% sucrose at −70 °C.

Hormones

[^{3}H] Ponasterone A ([^{3}H]Pon A; 180 Ci/mmol) was prepared by the reductive tritiation of the stachysterone precursor (see Dinan, 1985), and was kindly provided by C. Beckers (Technische Hochschule Darmstadt, F.R.G.). The radioligand was re-purified from autoradiolytic products every 6–8 weeks by reversed-phase high performance liquid chromatography (HPLC). Unlabeled ponasterone A (Pon A) was kindly provided by D.H.S. Horn (CSIRO, Australia), and 20-OH ecdysone purchased from Rohto Pharm. (Osaka, Japan). Hormones were HPLC purified previous to use.

Receptor binding assays

Animal samples were weighed and homogenized in ice-cold TE (10 mM Tris pH 7.4, 1.25 mM EDTA) buffer at a concentration of 400 mg/ml in a ground glass homogenizer (Kontes). Tissue samples were dissected from 250–300 animals and prepared as described for whole animals for each assay. Addition of 5 mM $MgCl_2$, 7 mM dithiothreitol, protease inhibitors (phenylmethylsulfonyl fluoride or tryasol), high salt buffer with detergent (400 mM KCl, 0.1% NP-40) or sonication after homogenization failed to effect increased receptor binding. Homogenization in TE was therefore considered to result in total cellular receptor extraction, and this procedure was used unless otherwise noted. Homogenates were centrifuged for 10 min at $15000 \times g$ and the supernatant re-centrifuged for 5 min. Receptor binding was determined in 0.1 ml aliquots of the second supernatant, with an additional aliquot taken to determine protein concentration using the BioRad microassay method. Sample preparations were usually 10–15 mg protein/ml.

Scatchard (1949) analysis was derived from competition studies where 0.15 pmol [^{3}H]Pon A (180 Ci/mmol) and increasing amounts of unlabeled Pon A were added to replicate 0.1 ml aliquots. In duplicate samples 100-fold excess unlabeled Pon A was added to determine non-specific binding. Maximal binding was determined in single point assays by adding 24 pmol [^{3}H]Pon A (30 Ci/mmol) to 0.1 ml aliquots to determine total binding, with 100-fold excess unlabeled Pon A added to duplicate samples to determine non-specific binding. In all studies, specific binding represents the difference between total and non-specific binding.

A preliminary time course study indicated maximum binding was attained between 1 and 2 h at 25 °C, or overnight at 4 °C. Incubations were therefore done for 2 h at 25 °C, with gentle vortexing after 1 h. Bound and free radioligand were separated by dilution of assay samples with 0.5 ml 5% activated charcoal (Serva), 0.5% dextran (Pharmacia) in TE buffer at 4 °C as modified from McGuire (1975). After incubation and centrifugation receptor-bound radioligand in the supernatant was dissolved in Hydrofluor (National Diagnostics) and quantified by liquid scintillation spectrometry. Counting efficiency was determined by the external standard-channel ratios method. Receptor concentration from all studies was determined by a modification of the statistical computer analysis of Zivin and Waud (1982). Dissociation constants (K_d) derived from competitive binding assays using radioligand concentrations below the apparent K_d were calculated according to Rodbard (1973).

Chromatography

Bound and free radioligand were separated in some experiments by Sephadex G-25 (Pharmacia) column chromatography with elution of 1 ml fractions in TE quantified by liquid scintillation spectrometry as described. Samples homogenized in high-salt conditions were de-salted by G-25 spun-column centrifugation (Neal and Florini, 1973) after a 10 min incubation at 4° C.

Results

Ecdysteroid receptor identification

The identification and characterization of ecdysteroid receptors in adult fat body was hindered by the inability to mass isolate this tissue due to its ubiquitousness and very fragile nature. We reasoned that its relative abundance, and its being the major known 20-OH ecdysone-responsive tissue in *D. melanogaster* adults, would allow a correlation of whole animal receptor concentrations to hormone-induced fat body functions. As described further on, whole animal receptor studies were then correlated to single assays of dissected tissue enriched for fat body.

Identification of ecdysteroid receptor in *D. melanogaster* tissues and cell lines has depended upon binding studies using the 20-OH ecdysone analog, Pon A, which can be radiolabeled with high specific activity (Dinan, 1985) and has a high affinity for the ecdysteroid receptor which is reflected by an increased physiological sensitivity relative to 20-OH ecdysone (Yund and Osterbur, 1985). In this study, the association of [^{3}H]Pon A with soluble binding moieties from 4- to 5-day whole female extract reached kinetic equilibrium at 1–2 h at 25° C or 16–20 h at 4° C (data not shown). The integrity of the radioligand after binding was determined by Sephadex G-25 separation of bound radioligand, with subsequent methanol and butanol extraction of the radioligand. Fig. 1 demonstrated co-elution of nearly all the extracted radioligand with unlabeled marker

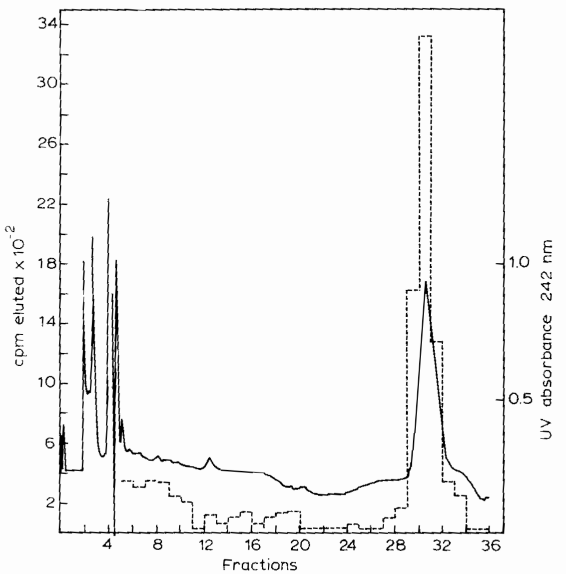

Fig. 1. Radioligand integrity after binding. [^{3}H]Pon A radioligand was incubated with a 4- to 6-day female extract, with the bound fraction separated by Sephadex G-25 chromatography and the radioligand extracted with methanol and butanol-water. Final butanol phase and concentrated and co-injected with unlabeled purified Pon A into reversed-phase HPLC. Eluted 1 ml fractions were monitored by UV absorbance (continuous line) and radioactivity counted (broken line).

Pon A after reversed-phase HPLC indicating receptor binding only to unmetabolized intact radioligand.

Steroid receptors have been found primarily in the nuclei from imaginal discs (Yund et al., 1978), but in both nuclear and cytosolic fractions from an embryonic cell line (Sage et al., 1982). The solubilization of nuclear proteins in whole animal extracts in the presence of high salt (0.4 M KCl) and detergent (0.1% NP-40), with subsequent de-salting previous to binding incubation, failed to increase specific binding, nor was significant specific binding detected in high-salt–detergent-extracted pellets (data not shown). These results indicate either a lack of extensive nuclear receptor accumulation, or solubilization of total cellular receptor with the TE homogenization procedure.

Ecdysteroid receptor characterization

Scatchard analysis of binding assays for 3- to 4-day females indicated two binding components (Fig. 2), with one having a dissociation constant of 2.94×10^{-9} M and a higher affinity component

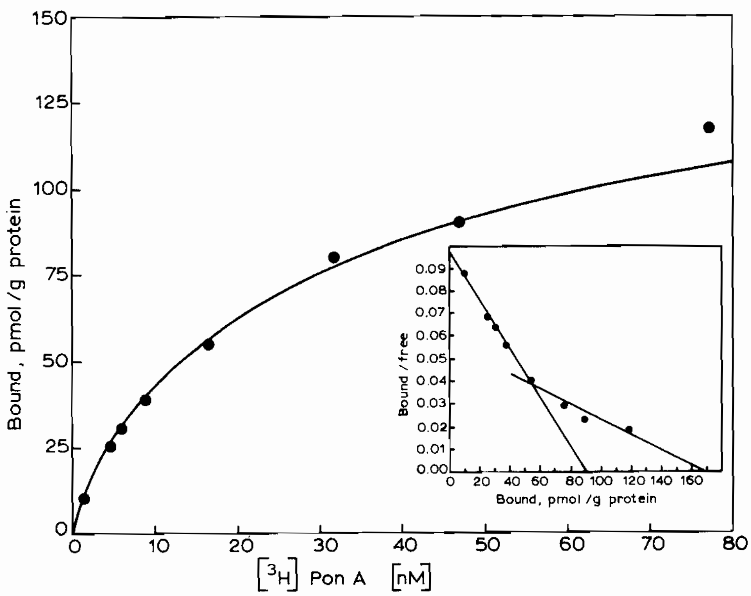

Fig. 2. Binding of increasing concentrations of [^{3}H]Pon A to 4-day whole animal extracts of adult females. Each data point represents the mean value of specific binding in two replicate assays (total binding less non-specific binding determined in replicate assays with 200-fold excess unlabeled Pon A). The inset is a Scatchard analysis plot of the binding data. Lines were plotted according to linear regression analysis with r values of 0.95 or greater.

with a K_d of 9.2×10^{-10} M (Table 1) and a maximum binding (B_{max}) concentration of 90 pmol/g protein. Specificity of the receptor for ligand was demonstrated by competition studies with unlabeled Pon A and the natural ligand, 20-OH ecdysone (Fig. 3). At the 50% binding level, Pon A was almost 13-fold more efficient in

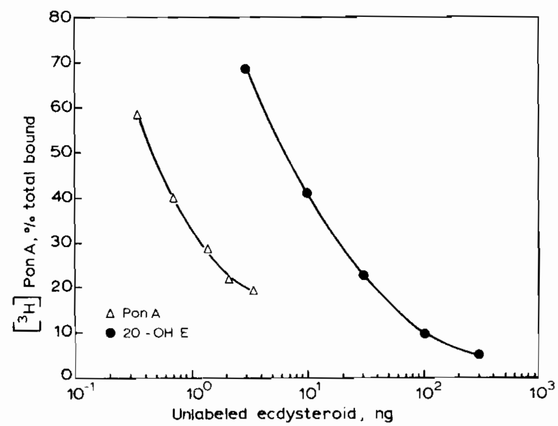

Fig. 3. Competitive binding to [^{3}H]Pon A with Pon A (Δ) and 20-OH ecdysone (●). Specific binding of 4-day female extracts incubated with 0.15 pmol radioligand and increasing concentrations of unlabeled ecdysteroids given as a percentage of total binding in the absence of unlabeled ecdysteroids.

competing for radioligand, which is consistent with the derived K_d for 20-OH ecdysone at 2.9×10^{-8} M (Table 1), and with previous competition studies (Maroy et al., 1978; Deak et al., 1988).

The macromolecular nature of the binding moiety was demonstrated by Sephadex G-25 gel filtration (data not shown) and a proteinaceous characteristic was suggested by heat lability studies. Receptor preparations from 1- to 2-day females were pre-incubated for 1 h at either 4°C or 40°C previous to radioligand binding. Almost all specific binding was lost after heat treatment ($B_{max} = 8.5 \pm 0.1$ pmol/0.1 pmol/g protein), while non-heat-treated samples maintained specific binding consistent with non-incubated samples ($B_{max} = 99$ pmol/g protein).

TABLE 1

BINDING PARAMETERS FROM COMPETITIVE BINDING STUDIES

Hormone	K_d	B_{max}
Ponasterone A	9.2×10^{-10} M	90 pmol/g
Ponasterone A	2.94×10^{-9} M	167 pmol/g
20-OH ecdysone	2.9×10^{-8} M	–

Receptor tissue specificity

To further identify the focus of the ecdysteroid receptors in adult females, and specifically to determine whether this could be localized to adult fat body, receptor assays were performed in dissected body segments and tissues. Since much of the fat body is attached to the abdominal integument, assays were performed in isolated abdomens containing all abdominal organs, and in dissected abdominal walls containing fat body as well as muscle and epidermal tissue. Table 2 shows that isolated abdomens yielded somewhat lower receptor concentrations compared to whole females assayed by single point determinations, although abdominal wall samples yielded receptor concentrations closer to whole animal levels.

A major tissue which could account for abdominal receptors is the ovary since embryonic receptors have been identified in oviposited eggs (Osterbur and Yund, 1982; Deak et al., 1988). Assays were performed on 3- to 4-day vitellogenic ovaries, the ovariectomized carcasses of the females from which they were dissected, and in females from a dominant female-sterile strain (*Fs(1)-K1237*) which is lacking in morphologically detectable ovarioles. Table 2 shows that mature ovaries contained a relatively low receptor concentration. In contrast, the ovariectomized females contained nearly a 5-fold higher receptor concentration compared to ovaries, which was slightly higher than the level measured in unoperated sibling females. Consistent with these results, the receptor concentration in genetically ovariectomized *Fs(1)-K1237* females was similar to unoperated vitellogenic females.

TABLE 2

ECDYSTEROID RECEPTOR CONCENTRATIONS

Sample	n [a]	Concentration (pmol/g) [b]
Unoperated females 4-day	4	122 ± 18
Female abdomens	4	62 ± 11
Female abdominal walls	2	104
Mature ovaries 4-day	3	24 ± 7
Ovariectomized females	2	168
Fs(1)K1237 females	2	112

[a] n-number of samples; two specific-binding determinations per sample.

[b] Mean value of ecdysteroid receptors/g protein ± SEM.

Discussion

20-OH ecdysone clearly has an experimental stimulatory influence on YP synthesis in *D. melanogaster*, yet ecdysteroid concentrations are relatively low in adult females, at levels equivalent to those found in males (Handler, 1982; Bownes et al., 1984) which normally do not produce YP. While the ovaries have been suggested as the source of ecdysone in adult females (Schwartz et al., 1985), normal YP synthesis proceeds in their absence (Postlethwait et al., 1980; Belote et al., 1985) and no other endocrine source of ecdysone has been discovered. On the other hand, juvenile hormone similarly stimulates YP synthesis (Postlethwait and Handler, 1980), but has a known endocrine source in adults and occurs at a relatively high concentration, although titers are not sexually dimorphic (Bownes and Rembold, 1986). These results raise the question of the relative importance of 20-OH ecdysone to vitellogenesis in *Drosophila*. We have begun to clarify how 20-OH ecdysone influences YP synthesis by identifying and partially characterizing ecdysteroid receptors in adult females, and by determining that their binding characteristics are consistent with a physiological influence at the observed hormone concentration.

Utilizing the high-affinity Pon A radioligand, two binding components were found having dissociation kinetics consistent with saturable, high-affinity binding proteins, and which are comparable to receptor previously identified in embryonic and larval tissues (Yund et al., 1978; Osterbur and Yund, 1982; Sage et al., 1982; Dinan, 1985; Deak et al., 1988). Notably though, the higher affinity component in adult females had a K_d 3-fold higher than the lower affinity component. These multiple binding components may be due to tissue heterogeneity in the receptor preparations, or alternatively, they may have physiological functions in fat body tissue resulting in a modulation of responsiveness to hormone. Two binding components have also been reported for *Drosophila* tumorous cell lines, having an almost 10-fold difference in their dissociation rate constants (Dinan, 1985). A determination of whether multiple binding components exist specifically in adult fat body, and whether differential binding affinities affect YP gene responsiveness to hormone will require further analysis of receptor purified from homogeneous tissue.

Receptor specificity for hormone was determined by competition studies with Pon A and 20-OH ecdysone, indicating a 13-fold difference in the K_d between Pon A and the natural ligand. This result is consistent with previous binding data (Maroy et al., 1978; Deak et al., 1988) and accurately reflects the physiological ecdysteroid response to the two hormones (Yund and Osterbur, 1985). Importantly, the apparent K_d for 20-OH ecdysone derived from this study was 2.9×10^{-8} M, which is comparable to the baseline circulating hemolymph ecdysteroid titers found in adult females (2×10^{-8} M; Handler, 1982) suggesting that the receptor binding sites are occupied in adult females. It should be noted that this K_d, while comparable to the K_d for imaginal discs (Yund et al., 1978), is 10-fold higher compared to embryos (Osterbur and Yund, 1982) and embryonic (Sage et al., 1982) and tumorous (Dinan, 1985) cell lines.

These binding studies, taken with evidence for the macromolecular and proteinaceous nature of the binding moiety, indicate that the binding of [^{3}H]Pon A by whole adult female extracts occurs with high affinity, specificity, and is saturable, fulfilling the criteria for ecdysteroid binding to receptor molecules. The observed K_d for 20-OH ecdysone is consistent with the hormone having a physiological influence on YP synthesis at concentrations previously assayed in adult females (Handler, 1982; Bownes et al., 1984).

The premise that receptor found in whole females represents to a large extent receptor in adult fat body, was supported by the finding of similar receptor levels in whole females and in dissected abdominal walls which have attached to it much of the insect's fat body. Importantly, the only tissue in the abdominal wall known to have an ecdysteroid-responsive function in 3- to 4-day females is the fat body, although earlier in development dopa decarboxylase activity is stimulated in the epidermis (Kraminsky et al., 1980). Furthermore, the only known ecdysteroid-responsive activity which could account for hormone receptors in the fat body, or in the whole female, is the stimulation of YP synthesis. Although residual receptor may exist in the epidermis, receptor studies in males indicate a 3- to 5-fold lower receptor level in whole males and male body walls (unpublished observations) which is consistent with a sexually dimorphic response to 20-OH ecdysone in the fat body. If abdominal wall receptor is primarily localized in the epidermis a sexual dimorphism would not be expected.

We found that ovaries contained significant receptor levels, but at a concentration considerably lower than that found in ovariectomized females or abdominal walls, and at a concentration similar to that found in early embryos (Deak et al., 1982). Interestingly, the ovarian follicular epithelium is also a site of YP synthesis (Brennan et al., 1982), although physiological studies do not support a response to 20-OH ecdysone in this tissue (Jowett and Postlethwait, 1980). If the ovarian receptor is limited to embryonal functions, this may explain the lack of ecdysteroid regulation of YP synthesis in this organ.

Together, the data indicates that adult *D. melanogaster* females have ecdysteroid receptors with binding characteristics consistent with receptors found earlier in development, and consistent with the 10^{-8} M to 10^{-7} M ecdysteroid concentration found in females having a physiological influence on YP synthesis in the fat body. Since the sex specificity of YP synthesis appears to be a cell autonomous activity not directly regulated by systemic hormone titers (Handler, 1982; Belote et al., 1985; Postlethwait et al., 1986), differential receptor affinity or quantity may have an important influence on the sex-specific regulation of YP gene expression.

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